

Entrance and Growth of Lactic Acid Bacteria in Gas-Exchanged, Brined Cucumbers†

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Received 29 June 1981/Accepted 27 August 1981

Entrance of lactic acid bacteria into the interior of brined cucumbers was found to be greatly influenced by gas composition of the cucumbers before brining. Exchange of the internal gas of fresh cucumbers with O₂ resulted in absorption of bacteria into the subsequently brined fruit within a few hours. Bacteria were absorbed into nonexchanged cucumbers to a lesser extent. Little bacterial absorption occurred in N₂-exchanged cucumbers. Stomata of the cucumber skin appeared to be a likely port for bacterial entry. When *Pediococcus cerevisiae* or *Lactobacillus plantarum* cells were added to the brine of O₂-exchanged cucumbers, the respective cell types colonized in large numbers within intercellular spaces and vascular elements of mesocarp tissue during fermentation of the cucumbers. Implications of these observations, particularly with regard to bloater formation in brined cucumbers, are discussed.

Bloater formation in brine-fermented cucumbers has been attributed to the production of carbon dioxide and other gases by fermenting microorganisms (3, 4, 5). Samish et al. (11, 12) concluded that bacteria are present in small numbers in healthy cucumbers, but when the cucumbers are brined, the bacteria multiply and produce sufficient carbon dioxide to cause bloater damage. Etchells et al. (4) proposed that carbon dioxide is produced solely by microbial activity in the brine surrounding the cucumbers and that the carbon dioxide diffuses into the cucumbers from the brine and collects at points of structural weakness inside the cucumber tissues. Fleming et al. (10) reported that cucumber tissue is an important source of carbon dioxide.

Bloater formation can be prevented by purging carbon dioxide from fermenting cucumber brine (1, 2, 6, 9). Purging is now widely used by the United States pickle industry to prevent bloater formation.

Fleming et al. (8) found that exchange of the internal gas of fresh cucumbers with oxygen resulted in the brined cucumbers gaining a fully cured appearance within 1 or 2 days, as compared with several months for nonexchanged cucumbers. Furthermore, the oxygen-exchanged cucumbers apparently were able to resist higher levels of carbon dioxide than were nonexchanged cucumbers, without serious bloater damage.

These investigators suggested that oxygen in the oxygen-exchanged cucumbers was metabolized to carbon dioxide when the cucumbers were immersed in brine and that the carbon dioxide formed dissolved in the tissue fluid to a greater extent than the oxygen that it replaced, thus creating a vacuum within the tissue that drew brine into the cucumbers.

Experimental 80-bushel ($\approx 2.9\text{-m}^3$) wooden tanks were used to determine whether the oxygen exchange treatment might be adaptable to commercial use (Fleming et al., unpublished data). The oxygen-exchanged cucumbers were visually cured within a day or so after brining, duplicating our earlier (8) laboratory data. However, in contrast to our previous laboratory data, serious bloater damage occurred in the oxygen-exchanged cucumbers about 4 to 5 days after brining. The interior of the bloated cucumbers contained bacteria as evidenced by microscopic observation. We decided to investigate the possibility that the vacuum created in oxygen-exchanged cucumbers may have drawn bacteria into the fruit along with the surrounding brine.

The objective of this study was to determine the effects of oxygen and nitrogen as exchange gases of fresh cucumbers on the entrance, location, and growth of lactic acid bacteria in brine-fermented cucumbers.

MATERIALS AND METHODS

Cucumbers. Size no. 3 pickling cucumbers (3.8 to 5.1 cm in diameter) were obtained from a nearby

† Paper no. 6970 of the journal series of the North Carolina Agricultural Research Service, Raleigh.

commercial grower. Only cucumbers free of disease and physical defects were used.

Gas exchange of cucumbers. Cucumbers (1.9 kg) were washed, weighed, and packed into 3.8-liter (1 gallon) glass jars fitted with lids, a gas inlet, and a brine reservoir as described previously (7). Cucumbers were exposed to either nitrogen (N_2) or oxygen (O_2) at a metered rate of 300 ml/min for 1 h to exchange the internal atmosphere (7). Nonexchanged (air) cucumbers served as controls.

Brining. After gas exchange, brine was added through the reservoir into the jar, while gas flow continued to prevent air from entering the jar. Cover brines contained 10.6% (wt/vol) NaCl, except where indicated. Brine used for fermentation also contained 0.32% (vol/vol) glacial acetic acid and 1.0% (wt/vol) sodium acetate trihydrate to approximate conditions specified in the controlled fermentation procedure of Etchells et al. (2). Fermentation brines were purged with N_2 at a continuous flow rate of 5 ml/min to prevent cucumber bloating (6).

Microorganisms, inoculation, and enumeration. Brines were inoculated with cells (log phase) of *Pediococcus cerevisiae* FBB-39 or *Lactobacillus plantarum* WSO that were harvested by centrifugation (7,000 rpm for 10 min), washed twice with saline, and suspended in saline. Plate counts of inocula, brines, and cucumbers were on LBS agar (BBL Microbiology Systems, Cockeysville, Md.) and reported as the number of lactic acid bacteria per gram or milliliter. Cucumbers were rinsed thoroughly under running tap water, aseptically transferred to sterile blenders containing 100 ml of sterile water, and ground to a homogeneous slurry. Reported plate counts of cucumbers are mean values of replicate sets of cucumbers (two to three cucumbers per set) from replicate treatments.

Surface sterilization of cucumbers. Cucumbers were surface sterilized by immersion of the fruit in a 10% Clorox solution (0.525% sodium hypochlorite [wt/vol]) for 0.5 h. The cucumbers were removed from this solution and held under an ultraviolet lamp until the surface was dry. Sterility of the cucumber surfaces was confirmed by swabbing them with moistened, sterile swabs which were then streaked on MRS agar and VRB agar plates. Controls consisted of cucumbers that were not surface sterilized.

Scanning electron microscopy. Tissue sections (5 mm² by 1 mm thick) of fermented cucumbers were fixed in 3% glutaraldehyde in 0.1 M sodium acetate buffer, pH 5.5. Sections were dehydrated in ethanol and passed through a graded series of amyl acetate. Liquid carbon dioxide (CO_2) was used as an intermediate fluid for critical-point drying in a Ladd 28000 instrument. Tissue was gold coated in a Polaron E 5000 diode sputtering system and observed at various magnifications at 20 KeV with an ETEC autoscanner microscope. Linear and gamma operating modes were used.

RESULTS

Bacterial entrance and growth in gas-exchanged cucumbers. Exchange of the internal gas of fresh cucumbers with O_2 or N_2 influenced

the number of lactic acid bacteria enumerated from cucumbers that were subsequently held in brine inoculated with *P. cerevisiae* (Table 1). At 4 h after brining, O_2 -exchanged cucumbers had significantly ($P \leq 0.05$) more lactic acid bacteria than N_2 -exchanged cucumbers. Numbers of lactic acid bacteria for control (air) cucumbers were between those for O_2 - and N_2 -exchanged cucumbers. Although the numbers of bacteria increased after 28 h of incubation, the bacterial counts were in the same order: $O_2 > \text{air} > N_2$.

Coefficients of variation of bacterial counts among cucumbers for O_2 -exchanged and nonexchanged fruit were noticeably larger than that for N_2 -exchanged cucumbers. It should be emphasized that the bacterial counts for this experiment represented total lactic acid bacteria in or on the cucumbers. No attempt was made in the above experiments to distinguish counts for superficial or embedded bacteria.

Evidence was obtained in a separate experiment, however, which indicated that lactic acid bacteria penetrated to the interior of O_2 -exchanged, brine-inoculated cucumbers. The brined cucumbers were surface sterilized in 10% Clorox solution before blending. Oxygen-exchanged cucumbers contained ca. 2×10^2 bacteria per g, whereas no bacteria were detected in N_2 -exchanged and nonexchanged fruit (Table 2).

TABLE 1. Effect of exchange gas on lactic acid bacterial counts of cucumbers held in an inoculated brine^a

Exchange gas	CFU/g of cucumber ^b at following time (h):	
	4	28
O_2	7.1×10^5 * (134)	7.4×10^7 ‡ (115)
Air	1.6×10^5 *† (178)	8.6×10^5 § (62)
N_2	1.8×10^4 † (62)	2.0×10^6 (19)

^a Cucumbers were covered with brine containing 4.8×10^7 colony-forming units (CFU) of *P. cerevisiae* per ml immediately after gas exchange.

^b Values with a common superscript symbol are not significantly different ($P > 0.05$) by Duncan's new multiple range test; coefficient of variation is shown within parentheses.

TABLE 2. Effect of surface sterilization of gas-exchanged cucumbers from inoculated brine on numbers of lactic acid bacteria^a

Exchange gas	CFU/g of cucumber	
	Surface sterilized	Not sterilized
O_2	2.0×10^2	1.9×10^3
Air	<10	2.9×10^3
N_2	<10	2.0×10^2

^a Cucumbers were sampled 5 h after the addition of brine containing 1.4×10^6 colony-forming units (CFU) of *P. cerevisiae* per ml.

The time of culture addition to the brine affected the number of lactic acid bacteria in O_2 -exchanged and nonexchanged cucumbers during fermentation. When the culture was added in the initial cover brine of O_2 -exchanged cucumbers, more bacteria were in the fruit than in the brine 2 days after inoculation (Fig. 1). Nonexchanged cucumbers contained fewer bacteria than the brine after 2 days (Fig. 1). After 6 days, populations in the cucumbers and brine were similar for O_2 -exchanged and nonexchanged cucumbers. When the culture was added 24 h after brining, however, the number of bacteria in the cucumbers never reached that in the brine,

whether the cucumbers were O_2 exchanged or not (Fig. 2). Regardless of time of culture addition, O_2 -exchanged, brined cucumbers contained more bacteria during early stages of fermentation than nonexchanged cucumbers (Fig. 1 and 2).

Location of bacteria within brined cucumbers. Bacteria were frequently observed to be associated with stomates on the cucumber surface (Fig. 3) with both gas-exchanged and nonexchanged, brined cucumbers. The stomatal openings appeared to be large enough for bacterial entry. Subcutaneous pustules on fully fermented cucumbers were macroscopically visible

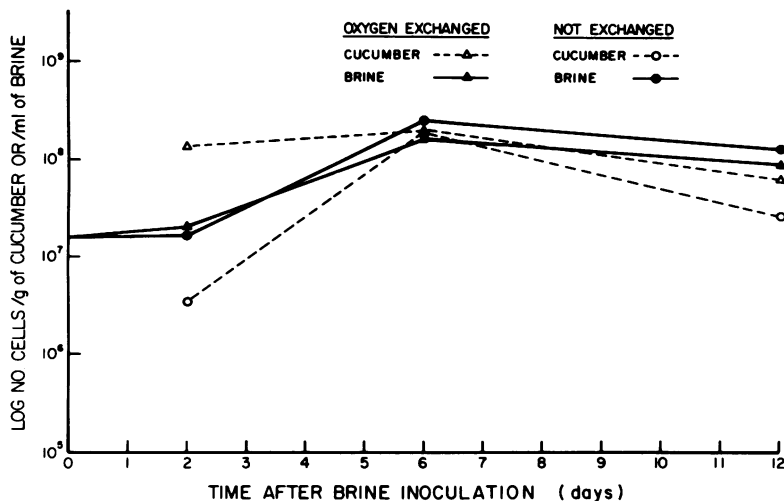


FIG. 1. Numbers of *P. cerevisiae* in the brine and in the cucumber during fermentation when the culture was initially present in the brine in which the cucumbers were placed.

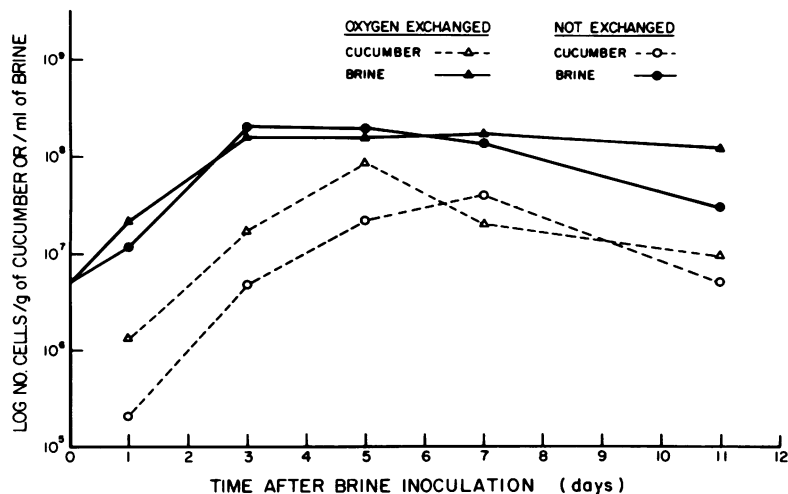


FIG. 2. Numbers of *P. cerevisiae* in the brine and in the cucumber during fermentation when the culture was added 24 h after the cucumbers were brined.

on the fruit surface (Fig. 4). With scanning electron microscopy, these pustules appeared as lens-shaped colonies formed directly below the epidermis (Fig. 5a). Closer examination (Fig. 5b) showed densely packed cells characteristic of *P. cerevisiae*, the brine inoculum. In a separate fermentation, *L. plantarum* was also observed in the pustules when this organism was the inoculum.

As viewed by scanning electron microscopy,

bacteria were localized in intercellular spaces in the mesocarp tissue (Fig. 5c and 5d) of O₂-exchanged cucumbers. Bacteria were also observed in nonexchanged cucumbers, but with less frequency. Vascular elements within the mesocarp were sites of bacterial colonies (Fig. 6a to d). The seed area (central core region) of the brined, O₂-exchanged cucumbers was nearly void of bacteria as seen microscopically.

Bacterial growth within O₂-exchanged tissue

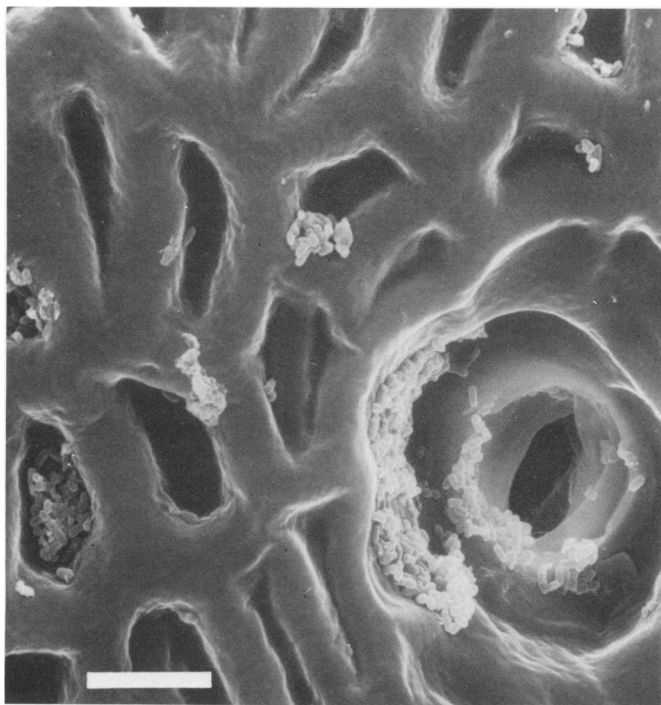


FIG. 3. Bacteria associated with a stomate on the surface of a cucumber fermented by *L. plantarum*. Bar, 10 μ m.

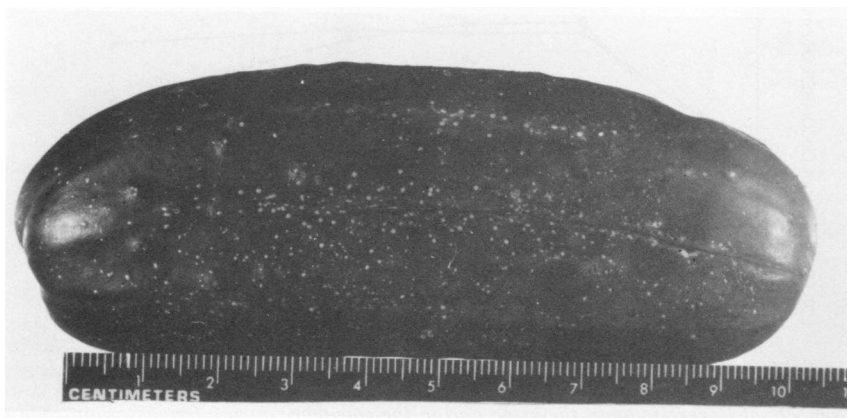


FIG. 4. Subcutaneous bacterial pustules on a fermented cucumber.

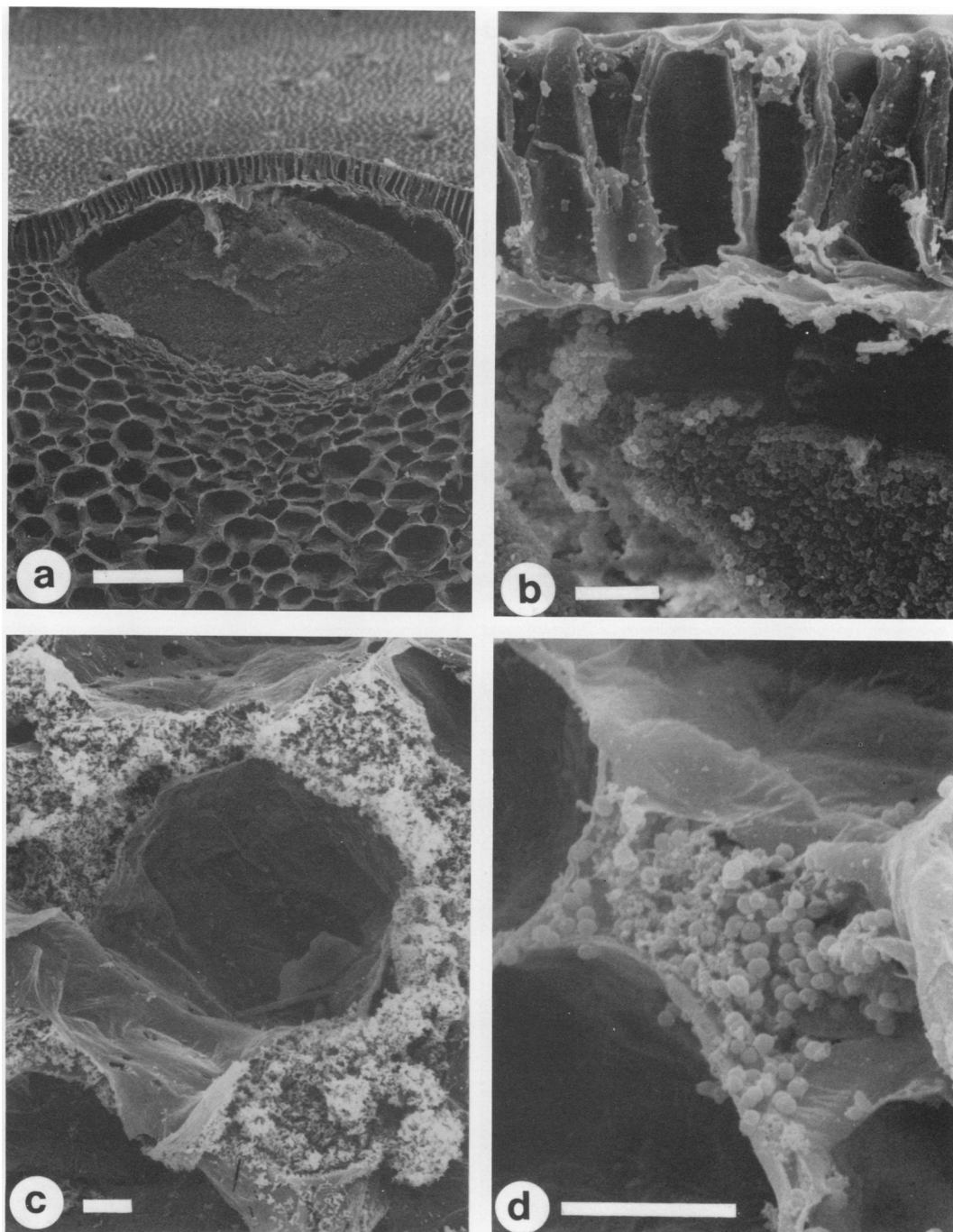


FIG. 5. (a) Cross-section of a bacterial pustule formed between the epidermal and mesocarp cells of a brined cucumber. The bacterium is *P. cerevisiae*; bar, 100 μm . (b) Larger magnification of (a); bar, 10 μm . (c and d) *Pediococci* within intercellular spaces of mesocarp tissue of a brined cucumber; bars, 10 μm .

also was evidenced by the presence of colonies that could be seen macroscopically (Fig. 7) or by light microscopic examination of a thin section

of the tissue. A milky liquid could be squeezed from O_2 -exchanged cucumbers that contained large numbers of bacterial cells. Not all cucum-

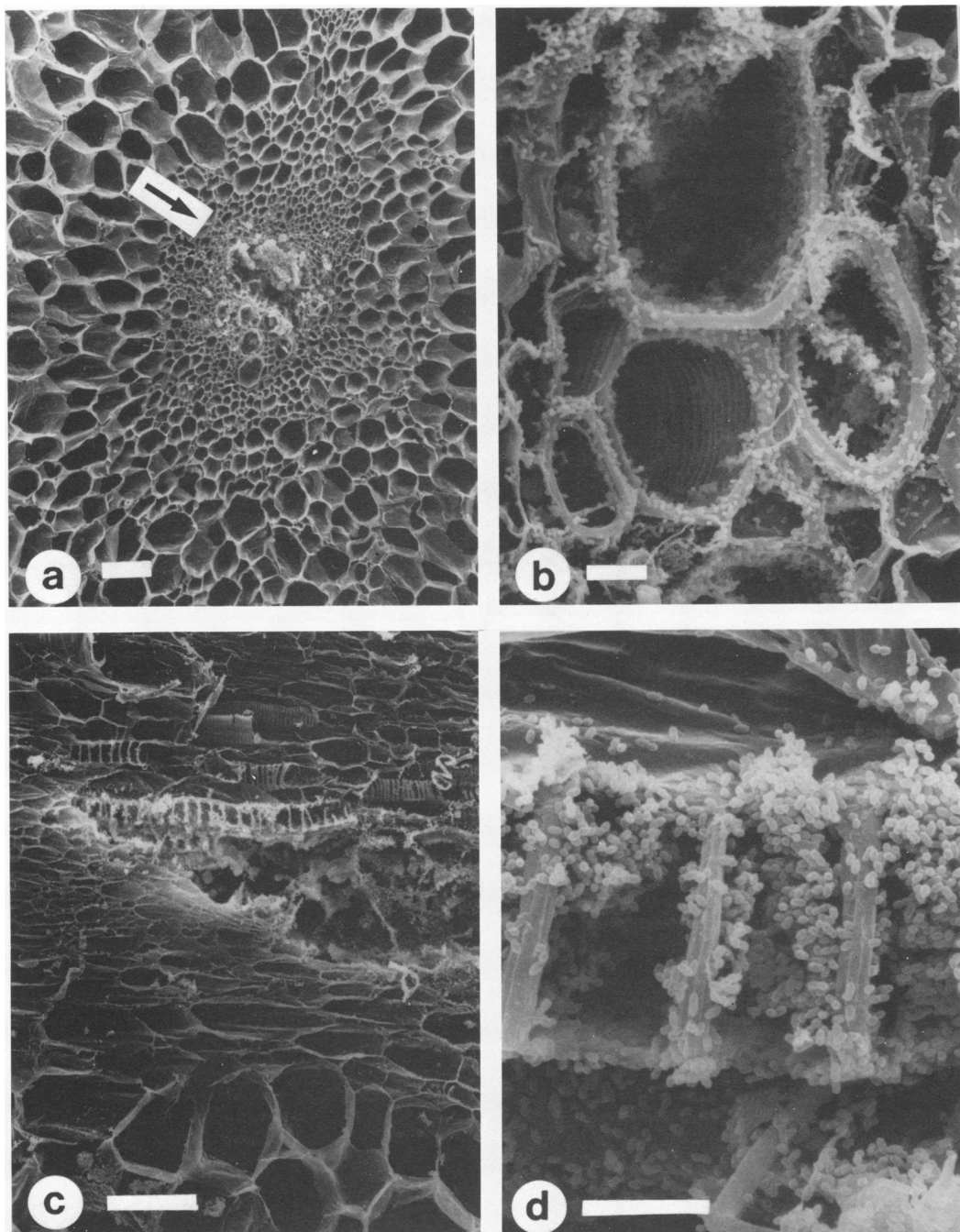


FIG. 6. Vascular elements in a brined cucumber colonized by a *Leuconostoc* sp. (a) Cross-section of vascular tissue (arrow); bar, 100 μ m. (b) Larger magnification of (a); bar, 10 μ m. (c) Longitudinal section of vascular tissue; bar, 100 μ m. (d) Larger magnification of (c); bar, 10 μ m.

bers appeared to be infected to the same extent, and the infection was not uniformly distributed throughout the flesh. Rather, colonization of bacteria appeared in discrete areas of the me-

socarp tissue and in vascular regions in some cases. In other instances, large areas of the flesh had a cloudy appearance due to widespread bacterial growth. No colonies were observed in

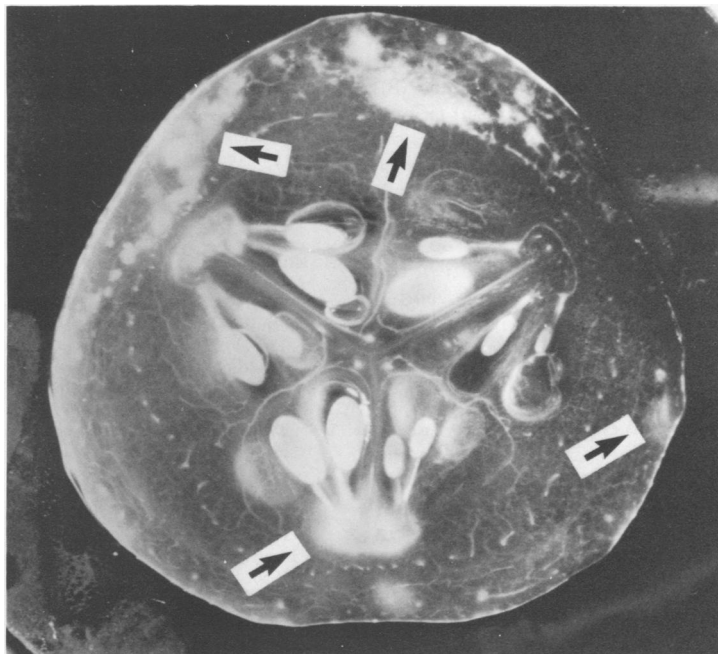


FIG. 7. Cross-section of an O_2 -exchanged, fermented cucumber. Arrows indicate areas of bacterial localization as evidenced by a milky appearance.

the seed area of O_2 -exchanged fruit. No visual colonies were present in the mesocarp of N_2 -exchanged or nonexchanged cucumbers.

DISCUSSION

We observed that lactic acid bacteria can enter brined cucumbers from the surrounding brine. Entrance into and extent of growth of the bacteria within the cucumbers was influenced by the internal gas composition of the cucumbers before they were brined. In O_2 -exchanged and brined cucumbers, entrance of bacteria was probably induced by the vacuum created within the fruit as a consequence of O_2 conversion to CO_2 . Fleming et al. (8) attributed the rapid appearance of visual cure (translucency) in the flesh of O_2 -exchanged and brined cucumbers to replacement of gas in intercellular spaces with brine. It seems possible, therefore, that bacteria may be entrained in the brine that enters O_2 -exchanged cucumbers. This logic is consistent with the observation that higher numbers of lactic acid bacteria were present with O_2 -exchanged cucumbers than within N_2 -exchanged or control (air) cucumbers (Table 1, Fig. 1).

Although the vacuum created in O_2 -exchanged and brined cucumbers may account for initial entrance of bacteria into the tissues, later bacterial entrance may be facilitated by the continuum of liquid from the surrounding brine

through intercellular spaces of the cucumber tissues. Conversely, it seems probable that gases trapped within nonexchanged cucumbers may serve as a barrier to entrance of bacteria into the brined fruit.

Nonexchanged cucumbers, however, may also be partially receptive to bacterial entrance according to the above reasoning. Fresh cucumbers contain ca. 20% O_2 , 75% N_2 , and 6% CO_2 (7). Conversion of the 20% O_2 to CO_2 with subsequent dissolution of the CO_2 may create sufficient vacuum to draw brine and bacteria into the outer tissue. In fact, greater numbers of bacteria were present in nonexchanged (air) than in N_2 -exchanged cucumbers that had been held in inoculated brine (Table 1).

Noticeably less variation existed among bacterial counts of N_2 -exchanged than of O_2 -exchanged and nonexchanged cucumbers; this is consistent with the thesis that numbers of bacteria that entered the N_2 -exchanged, brined fruit were relatively low. Variations in numbers of bacteria that entered O_2 -exchanged and nonexchanged cucumbers (Table 1) are assumed to reflect variations in the physical and physiological properties of the cucumbers. Stomatal frequency and degree of opening of stomatal guard cells (13), physiological state, moisture level, physical abrasions, and maturity of cucumbers may contribute to the receptiveness of the brined fruit to bacterial entrance.

Findings in the present study provide the basis for a hypothesis to explain why serious bloater damage did not occur when O₂-exchanged cucumbers were fermented in the laboratory (8), but did occur in a commercial experiment (Fleming et al., unpublished data). The cucumbers were carefully hand washed, and the cover brine and fermentation vessel probably contained relatively low numbers of naturally occurring bacteria in the laboratory study (8) compared with the commercial test. In the commercial test, the cucumbers were not washed, and the wooden tank and general handling procedure probably resulted in the higher numbers of naturally occurring bacteria which were absorbed by the O₂-exchanged and brined fruit.

In recent studies, we have been able to induce bloater formation in O₂-exchanged and brined cucumbers in the laboratory by inoculating the cover brine with lactic acid bacteria. Other types of bacteria might be even more efficacious in inducing bloater formation. We are continuing studies to explore numerous implications that have become apparent from the observations reported herein. In particular, we anticipate furthering the understanding of the mechanism of microbially induced bloater formation.

ACKNOWLEDGMENTS

We gratefully acknowledge Dorothy LaFave for laboratory assistance, K. R. Smith for providing one of the scanning electron micrographs, and R. L. Thompson for statistical assistance.

This investigation was supported by a research grant from Pickle Packers International, Inc., St. Charles, Ill.

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